Sustainable practices in Viticulture and Winemaking

Edited by: Costa, JM, Catarino S, Escalona JM, Comuzzo P. Elsevier.

Chapter 13

Microbiological control of wine production: new tools for new Challenges

M. Carmen Portillo and Albert Mas

Dept. Bioquímica i Biotecnologia, Facultat d'Enologia. Universitat Rovira i Virgili. 43007, Tarragona

Outline:

Abstract

Wine making has evolved since its origins in the Caucasus more than 8000 years ago to a modern scientific and technological discipline. Novel methodologies and practices have been implemented continuously in the elaboration of wines. The industry has been normally keen to accept those developments and incorporate them into their protocols. However, the complexity of some of the new developments, the "return" to old practices driven by some influencing wine makers or opinion-makers, commercial regulations and consumer concerns are growing limitations for the incorporation of new methodologies. This chapter is focused on new microbial

methodologies that can be applied to modern winemaking to control the process microbiologically and discuss about the possible challenges of their incorporation.

Key words: high-throughput sequencing; "omics" technologies; CRISPR/Cas 9; Spontaneous fermentation; SO_2 reduction

1. Introduction

Alcoholic fermentation (AF) is an essential step to produce any kind of wine in which the sugars present in the grapes (mainly glucose and fructose) are biotransformed by microorganisms to ethanol and carbon dioxide (Ribéreau-Gayon et al., 2006). In addition to sugars, grapes contain other compounds, like amino acids, polyphenols or acids, also susceptible of being metabolized and impact the flavour and aroma of the wine (Pretorius, 2016). The main microorganism of AF is the yeast *Saccharomyces cerevisiae* due to its adaptation to the harsh environmental conditions occurring during the winemaking process (low pH, high osmotic pressure, unbalanced concentrations of nutrients, high ethanol concentration, etc.) and its rapid transformation of sugars from the grape must. However, many other microbes including filamentous fungi, yeasts, and bacteria, are present during the winemaking process. The complex and highly diverse microbial communities associated with the fermentation of the grape must are known as wine microbiome.

Under certain conditions, some species of yeasts and bacteria can cause spoilage of the wine affecting its quality (Bartowsky, 2009). Wine susceptibility to spoilage depends on the species of yeast and bacteria present and their population size. Additionally, wine physical-chemical characteristics like ethanol content, residual sugar concentration, pH, amount and composition in main acids or oxygen, also condition wine spoilage (Bartowsky, 2009; Loureiro and Malfeito-Ferreira, 2003).

The microbiological stability of wine is fundamental to preserve its quality and produce sustainable wines avoiding economical losses. This stability may be achieved using chemical preservatives and/or physical treatments, aimed at killing microorganisms or at least at inhibiting their proliferation, or at physically removing them from wine by filtration. However, these treatments are not specific and may be detrimental for the desirable and beneficial microorganisms during fermentation. Thus, the very first step to control wine microbiome during wine production is to know its composition and its functional attributes.

In the last decade, a plethora of studies about wine microbiome have redefined our understanding of the microorganisms involved in the winemaking process. The combination of affordable high-throughput sequencing (HTS) technologies generating large datasets with insightful bioinformatic tools that enable analysis and interpretation of complex patterns has enhanced our understanding of wine microbiome composition and function. In particular, genomics, transcriptomics, metabolomics and proteomics have been broadly implemented to characterize microbial genes, transcripts and proteins, respectively, during wine production (Sirén et al., 2019a).

Recent rapid advances in HTS and DNA synthesis techniques are enabling the design and construction of new genes, gene networks and biosynthetic pathways and the redesign of cells and organisms for useful purposes (Pretorius, 2017). Additionally, with the advent of the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9) genome editing methods, yeast strain engineering has become rapid, efficient and multiplexed (Zhang et al., 2019).

This chapter will cover the role and future potential of such recent techniques in the microbial control of wine production and highlight the potential challenges that will be faced.

2. New Tools

2.1. "Omics" technologies: genomics, metagenomics, transcriptomics, metatranscriptomics, proteomics and metabolomics.

"Omics" technologies are primarily aimed at the universal detection of genes and transcribed genes in a single organism (genomics and transcriptomics, respectively) or in a microbiome (metagenomics and metatranscriptomics, respectively). Additionally, the term "omics" includes the technologies for the study of protein function, structure, and differential expression level (proteomics) and the metabolites generated from cellular processes (metabolomics) in a specific biological sample.

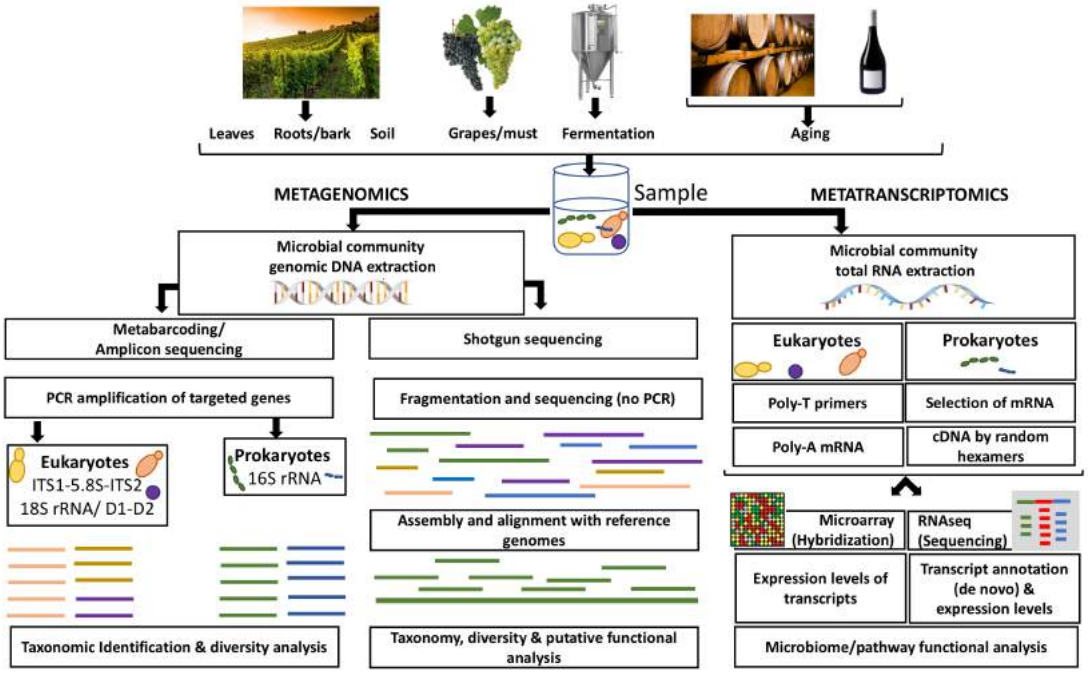

Figure 1: Schematic representation of the workflow followed during metagenomic and metatrascriptomic analysis from different wine-related samples. Within metabolomics, the comparison between metabarcoding and shotgun sequencing is presented, whereas the metatranscriptomics indicates the main differences between microarrays and RNAseq outputs.

Table 13.1. Summary of omics technologies applied to oenology. The asterisks indicate bibliographic review about the topic. Due to large number of articles in using metagenomics only reviews are indicated.

Omic technology	Target	Aim	References
Metagenomics	DNA	Microbial taxonomic identification, Genes, genetic pathways	Belda et al., 2020* Kioroglou et al., 2018* Stefanini and Cavalieri, 2018*
Metatranscriptomics	Total RNA and mRNA	Gene expression and functions	Alonso-del-Real et al., 2019 Barbosa et al., 2015 Curiel et al., 2017 Rossouw et al., 2015 Sadoudi et al., 2017 Shekhawat et al., 2019 Sunyer-Figueres et al., 2020 Tronchoni et al., 2017
Metaproteomics	Protein	Protein function, structure and differential expression level	González-Jiménez et al., 2020 Mencher et al., 2020 Peng et al., 2019
Metabolomics	Metabolites	Produced metabolites	Alañón et al., 2015 Alves et al., 2015 Arapitsas et al., 2018 Bordet et al., 2020 Cozzolino, 2016 Kioroglou et al., 2020 López-Malo et al., 2013 Mazzei et al., 2013 Peng et al., 2018 Petitgonnet et al., 2019 Richter et al., 2015 Roullier-Gall et al., 2020* Sirén et al., 2019a

The "Omics" analyses offer potential with regards to microbial control of wine production, and they have been applied in a plethora of wine related studies (Figure 1 and Table 1). Within the **metagenomics** analysis, the PCR amplification and the later sequencing of gene-marker specific regions is an approach known as metabarcoding or amplicon-based metagenomics. Alternatively, the shotgun sequencing retrieves the information from the whole metagenome of a sample (all genes from all genomes in the community) without including any primer selection and thus, is less biased by the PCR step. In fact, when comparing metabarcoding and shotgun metagenomics analysis of five spontaneous fermentations, metabarcoding analysis biased the overabundance of the genus *Metschnikowia* (Sternes et al., 2017). However, the combination of both metagenomic procedures has demonstrated to be useful to study the influence of vineyard community composition on the fermentation of Riesling and revealed the putative role of *Metschnikowia* as biocontrol agent against bacteria (Sirén et al., 2019b). Metagenomic analysis has created the notion that apart from LAB (Lactic Acid Bacteria) and AAB (Acetic Acid Bacteria), other bacteria, not previously described, may be present during the process (Godálová et al., 2016). Although the possible impact of these newly described bacterial genera is still to be demonstrated, Sirén et al. (2019b) detected an increase of functions assigned to class Actinobacteria at the end of fermentation, pointing to a putative role in winemaking. The metagenomic analysis has been mainly used to describe which microbes are present and relevant in wine-related samples, to reveal the relationship between the microbial communities and the wine terroir (reviewed in Belda et al., 2021), to monitor wine fermentations under different conditions (reviewed in Kioroglou et al., 2018), to relate the microbial communities with wine chemical composition (Bokulich et al., 2016) or to monitor the changes in the grape must and wine microbiota due to vineyard influence and different winemaking practices (Reviewed in Stefanini and Cavalieri, 2018). Another important question that metagenomic analysis has been called to answer, is whether grapes are the source of spoilage microorganisms (Renouf et al., 2005), or the wine-making equipment (Couto et al., 2005). Even though there is no clear answer to this debate, studies from Suárez et al. (2007) and Pinto et al. (2015) seem to support the latter hypothesis.

Knowing the composition of the microbial community during wine production is crucial to control it. That is the main reason why the first applications of "omics" techniques to wine research aimed the characterization of the bacterial, yeast and fungal communities. However, revealing the interactions of microbial communities in different stages of the

winemaking process and the metabolic pathways involved is of paramount importance to determine the microbial influence in wine quality. In this sense, metatranscriptomics, proteomics and metabolomics are the applied techniques to complement the metagenomics information.

One of the concerns that can be raised in metagenomics is that is a technology based on DNA. It is well known the resilience of this molecule and that can be present for long time after the microorganisms are dead. This could lead to an overestimation of the population. Some approaches have been proposed to tackle this aspect: one of them could be the use of RNA (see metatranscriptomics), known to be less stable and thus, could reflect the real live population and another possibility is the use of DNA binding dyes as ethidium monoazide (EMA) and propidium monoazide (PMA), which would prevent the amplification of DNA (Andorrà et al., 2010; Rizzotti et al., 2015; Navarro et al, 2020).

Metatranscriptomics refers to the measurement of total gene expression in a target sample by extracting messenger RNA (mRNA) and then converting it to cDNA using random hexamers or, in the case of Eukaryotes, poly-T primers that target the poly-A mRNA tail (Zepeda-Mendoza et al., 2018). This analysis gives information about the gene activity of the target organisms within the sample (Belda et al., 2017; De Filippis et al., 2018). Analyses can also be performed using stable isotope probing targeting an specific microbial group in the samples to enrich its transcriptome and then using the RNASeq in NGS platforms (Dumont et al., 2013). RNA-seq is the methodology that recently has become predominant in metatranscriptomics studies because it offers several advantages over microarrays. However, recent studies comparing both techniques pointed to the high consistence between both platforms, encouraging the use of microarray as a versatile tool for differential gene expression analysis (Nookaew et al., 2012). The metatranscriptomics analysis has been extensively used in wine research in recent years to elucidate, for example, interactions between microorganisms during wine alcoholic fermentation (Alonso-del-Real et al., 2019; Barbosa et al., 2015; Curiel et al., 2017), the effect of different stresses over gene transcription in wine microorganisms (Shekhawat et al., 2019; Tronchoni et al., 2017) or even to reveal the protective role of some compounds during the oxidative stress of wine yeasts (Sunyer-Figueres et al., 2020). Complete metabolic pathways are affected by altered gene expression, as shown by Sadoudi et al. (2017) with a change in acetic acid and glycerol metabolism in *S. cerevisiae* in the presence of *Metschnikowia pulcherrima*.

Furthermore, in the case of direct cell contact between two populations of distinct species, a change in the expression of FLO genes has been described, leading to a modification of population dynamics (Rossouw et al., 2015). The main challenge of the interpretation of the metatranscriptomics results during the study of yeast interactions, is that the growth of yeasts in mixed fermentations may be affected by several factors other than the specific used strains, as for example, the grape must composition, nutrient limitations, or fermentation temperature. All these factors should be considered to extrapolate the results from this analysis.

Metaproteomics is the identification and quantification of the expressed proteins in any matrix, which improves the functional gene annotations and provides better understanding of the microorganism interactions within that matrix. Generally, all mass spectrometry-based proteomic workflows comprise first the isolation of proteins from their source and can be further fractionated. After digestion, the peptides are analyzed by mass-spectrometry qualitatively and quantitatively. Then, the large amount of generated data is analyzed by appropriate software tools to deduce the amino acid sequence and, if applicable, to quantify the proteins in a sample. Recently, Peng et al. (2019) evaluated the proteomic response of *S. cerevisiae* during alcoholic fermentation when it was co-inoculated with *Lachancea thermotolerans*. Additionally, metaproteomics could be used to investigate the transcription of taste-active peptides in wine (González-Jiménez et al., 2020) or the possible involvement of extracellular vesicles in the complexity of wine sensory features (Mencher et al., 2020). Similarly to metatranscriptomics analysis, the biggest limitation of the metaproteomics technology is to evaluate the effect of the external factors over the results during of the experimentation making difficult the prediction of the transcriptome under the semi-industrial or industrial scale.

Metabolomics approaches aim to identify and quantify multiple metabolites or chemical compounds in a single matrix using nuclear magnetic resonance (NMR) or mass spectrometry-based methods (Cozzolino, 2016; Sirén et al., 2019a). Metabolomics data can provide general proof of gene function and complement the information gathered through metagenomics and transcriptomics studies. Both volatile and non-volatile metabolites can be studied in either targeted or non-targeted fashion. It is known that environmental factors and winemaking decisions have a strong impact on the microbial metabolic profiles and metabolomics is useful in the investigation of dynamics between microbial communities and the matrix (Cozzolino, 2016).

Metabolomics has already been applied to wine production to study questions ranging from the cultivar differences, monitoring of the fermentation process and guiding of winemaking decision making, as well as the exploration of aroma and flavor variation by vintage (Alañón et al., 2015; Arapitsas et al., 2018) or ageing conditions (Kioroglou et al., 2020). The literature includes various studies in which the specific composition of wine enables distinguishing between wines on the basis of fermentations with different yeast species and strains (Alves et al., 2015; López-Malo et al., 2013; Mazzei et al., 2013) and with single and co-cultures (Peng et al., 2018; Petitgonnet et al., 2019; Richter et al., 2015). Significant metabolic changes have been identified at each stage of the fermentation studied (Peng et al., 2018; Richter et al., 2015) highlighting that sampling time is an essential point for understanding interaction phenomena (reviewed in Bordet et al., 2020). Furthermore, some studies explore the differences between the endometabolome and the exometabolome associated with microorganisms involved in fermentation processes (Richter et al., 2015). It should also be noted that the identification of compounds detected during the metabolic profiling of wine remains difficult at present due to the incomplete that databases that frequently do not allow identifying all the biomarkers (Roullier-Gall et al., 2020).

2.2. Genome Editing: CRISPR/Cas9

The CRISPR/Cas9 genome editing tool has been successfully implemented both in *Saccharomyces* and non-*Saccharomyces* genome modification attempts and it is evident it will become more routine (Raschmanová et al., 2018). In short, CRISPR/Cas9 involves utilizing the natural mechanism that has been described in bacteria and archaea to develop a tool capable of conducting precise genome editing of any organism. Most CRISPR/Cas9 editing systems require two components i.e., a guide RNA, which is a chimeric RNA molecule, and an RNA-guided DNA endonuclease like Cas9. Part of the guide RNA is bound by the Cas9 and directs it to the complementary genomic DNA region causing a double strand break upstream of a protospacer adjacent region (that in the case of the commonly-used Cas9 from *Streptococcus pyogenes*, it is an NGG sequence). A double strand break would often be lethal for an organism if not repaired rapidly. The endogenous repair machineries allow for the introduction of a variety of genomic modifications. This tool has been fine-tuned and streamlined for yeast DNA editing (Jakočiunas et al., 2016; Weninger et al., 2018). Advantages of a CRISPR/Cas9 tool include that changing the target locus can be done simply by modifying a 20-bp sequence of the guide RNA and, once supplied with an appropriate

repair template, large insertions and deletions can be done. Also, the selection marker can easily be removed from the resulting strain, a great concern for any genetic modification in food applications. The genetic modification of wine strains of *Saccharomyces cerevisiae* has shown tremendous potential in improving many oenological aspects albeit mostly restricted to laboratory level (Van Wyk et al., 2019), as summarized in Table 2.

Table 13.2: Recent applications of the CRISPR-Cas9 technique in *Saccharomyces cerevisiae* to improve some aspects of wine making.

Wine making Goal	Gene edited	Result	Reference
Reduction of urea	*CAN1* (arginine permease)	25-40% urea reduction	Vigentini et al., 2017
Reduction of urea and ethyl carbamate	DUR3 (Urea transporter)	92% urea reduction 52% Ethyl carbamate	Wu et al., 2020
Fermentation of high sugar concentration and glycerol production	*STL1* (Sugar transporter)	Low fermentation activity, increased glycerol	Muysson et al., 2019
High glycerol production	*GPD1* (glycerol 3- phosphate dehydrogenase)	High production of glycerol	van Wyk et al., 2020
Aroma production (esters and acetates)	*ACT1* (alcohol acetyltransferase)	High concentration of several acetates	van Wyk et al., 2020

Wine yeasts are known to produce a broad array of compounds, not all of them with a positive character in wines. One of them is the generation of urea that can combine with ethanol and produce the carcinogenic compound ethyl carbamate. This has been the target of the first application of this technique in wine yeast. Vigentini et al. (2017) have eliminated the arginine permeases pathway (the *CAN1* gene) to reduce urea production in two different commercial strains of *Saccharomyces cerevisiae*. They have obtained reductions between 20 and 35%, depending on the strain. Reduction of urea has also been obtained by a different strategy using also the CRISPR-Cas9 editing tool: overexpression of the *DUR3* gene (Urea active transporter, Wu et al., 2020). They observed that the modified *S. cerevisiae* also reduced the level of urea by 92% and those of ethyl carbamate by 52% in Chinese rice wine.

Another successful application has focused on the glycerol response to high sugar concentration that is required in yeast fermenting special wines with this high sugar content. In this case, Muysson et al. (2019) deleted the functional *STL1* gene to analyze their effect in ice-wine fermentations and the resulting mutant yeasts presented reduced fermentation performance and elevated concentrations of glycerol and acetic acid,

compared to parental strains. It has to be emphasized that genes involved in ethanol and glycerol modulation will be the target for genetic modifications, in order to get wines with reduced alcohol content (Goold et al., 2017). In the same pathway (production of glycerol), van Wyk et al. (2020) overexpressed the gene GPD1(Glycerol 3-phosphate dehydrogenase) by changing the promoter. The resulting strain had significantly higher production of glycerol but also acetic acid that the parental strain. In the same work, they also focused on the production of aromas (acetate esters), overexpressing alcohol acetyltransferase (*ACT1*). The doble mutant had also increased levels of glycerol, and very high concentrations of the different acetates analyzed (ethyl acetate, isoamyl acetate, isobutyl acetate, phenylethyl acetate, hexyl acetate).

However, this technique is open to be used to many other non-*Saccharomyces* yeasts (Raschmanová et al., 2018). So far, its application to other yeast has been mostly for other applications (production of products with pharmaceutical or nutrition interest, or production of biofuel, for instance). Only the wine related yeast *Brettanomyces bruxellensis* has been successfully modified (Varela et al., 2020). However, the applicability of this modification is mostly in the brewing, as *Brettanomyces* is used for the development of some beer aromas.

Another interest in genome modification is to expand on the aroma-producing capabilities of wine yeast. This includes overexpressing genes involved in the synthesis of esters like the alcohol acetyltransferases 1 and 2, which promote increased condensation between alcohols and acetyl-CoA resulting in more acetate esters being produced (see above, work of van Wyk et al., 2020).

Despite some drawbacks, the value of the CRISPR/Cas9 tool in generating wine yeast strains remains largely unexploited. Of the current genome editing tools available, CRISPR/Cas9-based editing have been shown to be the most adaptable, versatile, and cost-effective. This methodology has opened a new era for the improvement and genetic modification of the wine yeasts. The process should be seen in two different ways, on one side to improve the knowledge acquisition but in another to improve wine quality. It is evident, though, consumer acceptance to these methodologies requires still a communicative effort with educational purposes from researchers and innovators. Legislation will probably follow the consumer's concerns but, most interestingly, it should be shifted to food safety, clearly stating the benefits and risks of using this methodology.

3. New Challenges

3.1. Grape microbiome and its control

Grape berries harbor a wide range of microbes including bacteria, fungi and yeasts originated from the vineyard environment (Zarraonaindia et al., 2015), many of which are recognized for their role in the must fermentation process shaping wine quality. Experimental analyses suggest that microbes colonizing berries could significantly affect grapevine and fruit health and development (Barata et al., 2012). Furthermore, grape microbiome also contribute to shaping phenotypic characteristics, such as flavor, color, and sugar content (Belda et al., 2017) thus influencing the winemaking process as well (Capozzi et al., 2015).

HTS techniques have being used to characterize bacterial communities of the grapevine plant (Belda et al., 2021) and to assess the provenance of some microbial groups (Bokulich et al., 2013; Zarraonaindia et al., 2015). It has been revealed that soil serves as a primary source of microorganisms with edaphic factors influencing the native grapevine microbiome (Zarraonaindia et al., 2015) and that the grape microbiome biogeography is non-randomly associated with regional, varietal, and climatic factors across multiscale viticultural zones (Bokulich et al., 2014). Moreover, Bokulich et al. (2016b) suggested a strong association involving grapevine microbiota, fermentation characteristics and wine chemical composition. The beneficial effect of certain microbial taxa on host plants as growth promoters and stress resistance inducers has been reported in several articles and some of them addressed their influence on grape and wine quality (Huang et al., 2018; Yang et al., 2016). Thus, the control of the grape microbiome through physic, chemical or biological treatment of the grapevine to promote certain taxa could affect both the health of the plant and the quality of the wine. Since microbiome metabolism can contribute to that of the plant host and the biochemical composition of its fruits, the nature of grapevine microbiome taxa identities, ecological attitudes, potential toxicity, and clinical relevance are all aspects worthy of a thorough investigation and the new technologies and tools explained in the section 2 are the most promising right now.

3.2.Reduction of SO_2 use

Sulfites are considered the main additives in winemaking for their antimicrobial and antioxidant activities. The most important role of this compound lies in its antimicrobial action against acetic and lactic acid bacteria, and molds to prevent spoilage and to determine the microbiological stabilization of wines to enhance aging potential. Furthermore, sulfur dioxide (SO_2) addition prior to the onset of alcoholic fermentation

340 also exerts a selective antimicrobial activity against spoilage yeasts, by inhibiting their
341 growth and promoting the rapid development of *Saccharomyces cerevisiae*. The current
342 concern about the potential negative effects of SO_2 on consumer health has motivated
343 the interest on replacing or reducing SO_2 use. Thus, research is focused on looking for
344 other preservatives and innovative technologies, harmless to health, to reduce SO_2
345 content in wine. Recently, numerous alternatives have been proposed to replace the
346 activity of SO_2 by the use of chemical additives and physical treatments, aimed at the
347 microbiological stability of wine (reviewed by Lisanti et al., 2019).
348 There are many different chemical solutions (antimicrobial compounds), some of them
349 approved by the EU authorities and/or OIV legislation. The most used chemical
350 alternative to SO_2 is the Dimethyl Dicarbonate (DMDC), which is active on the
351 inhibition of some microbial critical enzymes and is hydrolyzed to CO_2 and methanol. It
352 kills yeast cells almost immediately and later the residue is minimal, without any health
353 concern (Ribéreau-Gayon et al., 2006). The effectiveness of DMDC could be
354 jeopardized in musts with high microbial load, but it is considered very effective in final
355 wines, especially sweet and semisweet wines, once the viable load of microorganisms is
356 reduced (Bartowsky, 2009) The effect on bacteria is more limited, and when bacteria is
357 the main microbial problem, the use of lysozyme could be another alternative.
358 Lysozyme acts by hydrolysis of the cell wall in gram positive bacteria (for instance
359 lactic acid bacteria) but it does not have any action against gram negative bacteria (such
360 acetic acid bacteria) or yeast. Sorbic acid has been traditionally used in the food
361 industry as antifungal compound and in wines has been considered effective to inhibit
362 refermentation by *S. cerevisiae* in bottled sweet wines (Zoecklein et al., 1995) and
363 towards the growth of film-forming yeasts (*Candida* spp.) on the wine surface
364 (Ribéreau-Gayon et al., 2006). Nowadays it is hardly used for its limited effect and the
365 possible negative effects on consumer's health. Some of these treatments are not really
366 alternatives because of their limited microbial effects but are recommended to be used
367 together to reduce the SO_2 dosage (Ribéreau-Gayon et al., 2006).
368 Some other additives, also common in winemaking for other reasons are also known to
369 have some antimicrobial action against wine spoilage microorganisms. Among them,
370 we can mention the phenolic compounds (Silva et al., 2018) or chitosan (Ferreira et al.,
371 2013; Valera et al., 2017). Due to the interest to reduce or eliminate the use of SO_2,
372 many other compounds are being tested, although they are not yet authorized in the EU.
373 Among them, we can mention nisin, basically for the treatments against Lactic Acid

374 Bacteria (Rojo-Bezares et al., 2007), silver nanomaterials, active against yeasts, LAB
375 and AAB (Garde-Cerdán et al., 2014) or hydroxytyrosol active also against the three
376 kinds of wine microorganisms (Ruiz-Moreno et al., 2015). Finally, saturated short-chain
377 fatty acids were also used to control the growth of some spoilage yeasts (Ribéreau-
378 Gayon et al., 2006).
379 An option that is gaining interest is the use of some microorganisms able to inhibit the
380 growth of other microorganisms through several mechanisms, among them, cell-to-cell
381 contact (Nissen and Arneborg, 2003) or antimicrobial peptides (Albergaria et al., 2010).
382 This option is named as biocontrol. In fact, it has been described that the interaction
383 between yeasts induces the Viable But Not Culturable states as a mechanism to
384 overgrow the other yeasts and take over the alcoholic fermentation (Branco et al., 2015;
385 Wang et al., 2016). Even *S. cerevisiae* can enter this state in presence of other non-
386 *Saccharomyces* species (Navarro et al., 2020). Thus, biocontrol, or the use of certain
387 yeasts to limit the growth of others is a very attractive line of research.
388 Furthermore, some other alternatives for microbial stabilization have been considered,
389 mostly physical treatments. Among them, microfiltration is probably the most useful at
390 cellar level. However, several concerns have been raised regarding wine quality as
391 microfiltration will also remove colorant matter other macromolecules and even volatile
392 compounds, which will be very detrimental for wine quality due to its sensory impact
393 (Lisanti et al., 2019). Thermal treatments are also a possibility, although their impact on
394 sensory attributes limits its application to low quality wines exclusively (Ribéreau-
395 Gayon et al., 2006). Other physical methods, such as high hydrostatic pressure,
396 ultrasound, pulsed electric fields, ultraviolet irradiation, and microwave, successfully
397 used in the last few years for the microbiological stabilization of wine as alternative to
398 the use of SO_2 should be considered still far from a routine use in cellars.
399 Although exhibiting a certain microbial inhibition, no physical or chemical treatment
400 has to date shown to be able to replace the efficiency and the broad spectrum of
401 antimicrobial action of SO_2 (Santos et al., 2012). Thus, the main challenge when
402 reducing SO_2 or substituting it by chemical compounds or physical treatments would be
403 the microbial control during and after fermentation in addition to the control of the
404 organoleptic properties of the produced wine.
405 The improvement of the tools for microbial monitoring described in the previous
406 sections, could be good help for the microbial control. However, those tools are still far
407 from being useful at cellar level, as they are costly, time-consuming and with complex

interpretation. Adequation of those methodologies to cellar level is far from being practical, although it might be a research and transfer objective.

3.3.Spontaneous vs inoculated fermentations

Traditionally, alcoholic fermentations have proceeded spontaneously, with the microbiota that was already present on the grapes or resident in the winery. The spontaneous fermentations are normally slow and with unpredictable outcome, as it depends on the microbiota present and its capability to overcome the other yeasts. The wine is normally considered that reflects the "terroir" typicality, but it might have many risks of spoilage. The control of all fermentative processes is normally done by starter cultures, that could be from a fermenting substrate or pure microbiological cultures. Thus, in wine making we might have spontaneous fermentation (without any starter culture) or inoculated fermentations when a starter is used.

Either a fermenting substrate or from pure microbiological culture the inoculation has been traditionally done by pied-de-cuve. In those cases, the name comes from the "bottom of the deposit" that means that a 5-10 % of the total volume of the deposit is filled with an actively fermenting must and the rest of the deposit is filled up with fresh must. In this way, as the fermenting must has a very high concentration of yeast that are very active (typically could be between 10^7-10^8 cells/ml) could easily take over the fermentation of the whole deposit (the population reduction of one log unit is not relevant, as yeasts are already active and growing). With this mechanism the winemakers ensure a quick fermentation start and a good rate. If the pied-de-cuve is derived from a single culture (normally a selected yeast strain), this yeast must be propagated in optimal culture medium until it achieves a volume that can be used as pied-de-cuve. Often the last passages are done with must either sterilized or with low indigenous population. In this way, the selected strain will take over the fermentation and provide the final wine with the characteristics that the strain can develop in the wine, although this is not the case with the pied-de-cuve from fermenting vats, as they are the result of a mixed inoculum. However, the propagation needs a laboratory where minimal sterile conditions could be kept as well as it is a slow process that may take several days.

During the last Century many different strains of *S. cerevisiae* have been selected to be used as starter cultures to repress the wild microorganisms and achieve more predictable and desired outcomes. A big step forward in the use of starter cultures was the development of the Active Dry Wine Yeasts, where yeasts are dehydrated maintaining

their full activity, that is restored quickly after rehydration (Fleet, 1993). This must be considered a cellar-friendly procedure, as yeasts could be rehydrated in less than 30 minutes in the same cellar, facilitating the seeding of high numbers of yeast cells that are fully active and can initiate the alcoholic fermentation quickly and effectively. In this way, the fermentation proceeds very fast and with good fermentation rate (Figure 2).

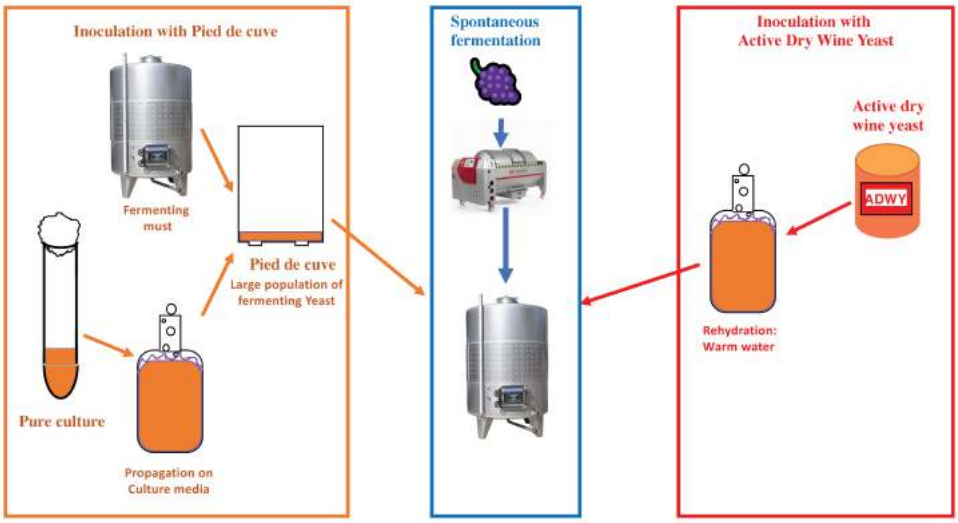

Figure 2: Inoculated and spontaneous alcoholic fermentations.

However, these inoculated fermentations present the risk of uniformity, as selected yeasts provide a limited diversity of the final wines (Fleet, 1993). Against this "uniformization" several strategies have been in use: selection of local yeasts or mixed inoculation with selected non-*Saccharomyces* yeast. Recent movements of non-conventional wine making (organic, biodynamic, natural, etc.) have challenged the use of Active Dry Yeast. A good alternative in these cases could be the use of pied-de-cuve that can be derived from small volumes of fermenting early musts that could be selected according to some variables (good fermentation activity and good sensory attributes), although the microbiological control will not be optimal, as there will be a mixed microbial population.

Grapes harbor a complex microbial community of fungi, bacteria and between 10^4 - 10^6 yeasts cells per gram of grapes (Fleet, 2003), which are mainly non-*Saccharomyces* yeasts. The populations of *Saccharomyces* are indeed very low in grapes (Beltran et al., 2002). These populations change when they enter in contact with the cellar environment where they join the resident microbiota. In fact, the cellar is a good niche for *S. cerevisiae*, which becomes the main cellar-resident yeast (Beltran et al., 2002). Although the grape must is a very complex medium and can provide support for many different microorganisms, there are some characteristics that transform such universal medium into a very restrictive one. The high sugar concentration, that derives in high osmotic pressure and low water activity; the high concentration of organic acids, with pH between 3 and 4 and the unbalance between nitrogen carbon sources makes the grape must a very selective medium. Thus, the initial grape juice only supports the growth of certain microbial species favoring the development of fermentative yeasts. Overall, species of *Hanseniaspora*, *Candida* and *Metschnikowia* genera begin the fermentation process. Species of *Pichia*, *Issatchenkia* and *Kluyveromyces* can also develop during this stage. These yeasts species may grow up to 10^6-10^7 cfu / mL of must until mid-fermentation when their population sharply decay. At this moment, *S. cerevisiae* becomes predominant, reaching populations of 10^7-10^8 cfu / mL, until the fermentation is completed. Nevertheless, the microbial succession occasionally can lead to stuck or sluggish fermentations as a result of an excessive proliferation of non-fermentative yeasts that consume nutrients needed for the development of the fermentative ones (Ciani et al., 2006; Medina et al., 2012; Padilla et al., 2016). Thus, the inoculation of *Saccharomyces* starters is a tool for the wine maker to define wine production and quality. However, this practice leads to the uniformity of the produced wines and some new tendencies in winemaking tend to prevent the use of standard commercial starters. In fermentations without use of starters (spontaneous fermentations), the native microbiota, mostly non-*Saccharomyces*, proliferate for several days, producing different compounds that could improve the organoleptic quality of the wines, although it also includes a risk of spoilage and sluggish or stuck fermentations. The improvement has been correlated to the presence of interesting enzymatic activities, some of them of technological interest (pectinolytic activities that facilitate procedures in the cellar) or to improve the final wine (esterases, beta-glucosidase, etc.) (Jolly et al., 2014). Additionally, these Non-*Saccharomyces* yeast may be able to reduce ethanol (Gonzalez et al., 2013), which has been proposed as a key

objective in current winemaking due to the increased concentration of sugars, among other effects, derived from climate change (Mira de Orduña, 2010). Nevertheless, the return to spontaneous wine fermentations may have considerable drawbacks especially in terms of economic losses, as these wines have much higher risks of presenting different levels of spoilage (presence of unwanted compounds that will be organoleptically detectable) that will not be acceptable for the consumer. Alternative microbial starters used in mixed or sequential fermentations, mainly non-*Saccharomyces*, have received increasing attention for their potential to produce wines with more distinctive and typical features (Jolly et al., 2014). This topic will be covered in the next section.

In order to take advantages of both inoculated and spontaneous fermentations and to improve certain wine characteristics, mixed and sequential fermentations using *S. cerevisiae* and different yeast strains or malolactic bacteria have attracted recent research interest (reviewed in Petruzzi et al., 2017). For example, it has been shown that *Torulaspora delbrueckii* enhances the complexity and fruity notes of wines (Renault et al., 2015) *Hanseniaspora vineae* enriches wines with fruity and flowery aromas (Lleixà et al., 2016b), *Lachancea thermotolerans* increases the total acidity (Gobbi et al., 2013) and *Metschnikowia pulcherrima* reduces the ethanol levels and enhances varietal aromas (Medina et al., 2012; Quirós et al., 2014). The increasing number of species used, often associated to new isolations from spontaneous fermentations (Garofalo et al., 2015; Padilla et al., 2016) introduces a relevant challenge in terms of interspecific interactions (Ciani and Comitini, 2015; Tronchoni et al., 2017; Wang et al., 2016). For example, to optimize the use of non-*Saccharomyces* yeasts or bacteria in mixed or sequential fermentations with *Saccharomyces* spp., is necessary to better understand their metabolism and nutrient requirements. During a sequential inoculation, the initial consumption of nutrients by non-*Saccharomyces* yeasts could affect the growth and survival of *Saccharomyces* yeasts, inoculated later (Lleixà et al., 2016a; Medina et al., 2012; Roca-Mesa et al., 2020). Furthermore, we have to consider that different grape varieties and batch volumes could influence the growth and final biomass of yeasts in mixed fermentations (Gobbi et al., 2013; Padilla et al., 2017). Thus, the field of interspecific interactions is of particular interest and necessary to scale from laboratory to industrial or semi-industrial scale.

3.4.The search for new strains

More than two hundred commercial strains of wine yeast available on the market are used by winemakers to produce different types, varieties, and brands of wines. However, due to the highly competitive wine market with new demands for improved wine quality, it has become increasingly critical to develop new wine strains (Bisson, 2004).

Besides the isolations of new species of yeasts mainly from spontaneous fermentations (Garofalo et al., 2015; Padilla et al., 2016; Torija et al., 2001), the new genetic tools allow the metabolic engineering of known strains. Classical strain improvement methods based on the repeated alternation of successive stages of mutagenesis and selection have frequently been used to obtain starter cultures of wine strains (Steensels et al., 2014). These methods are quite lengthy and time-consuming because require screening of a significant number of isolates. In fact, they have now been replaced by adaptive or directed laboratory evolution methods (ALE) which are more targeted and convenient (Sandberg et al., 2019). ALE technique is based on the selection of candidate strains through serial or continuous culturing of a particular yeast strain for many generations under selective pressure (i.e. high ethanol or high osmolarity) and has been applied successfully in previous studies (Betlej et al., 2020; Kutyna et al., 2012; McBryde et al., 2006).

Recently, novel methodologies for precise wine strain engineering based on better molecular knowledge have emerged due to the rapid progress in genomic studies with wine yeast strains, especially in *S. cerevisiae* strains (reviewed in Eldarov and Mardanov, 2020). An example of this new approaches would be the CRISPR/Cas9 tool. Nevertheless, when taking in consideration the real commercial implementation of all these and other advances, a barrier arises: engineered yeasts are usually considered genetically modified organisms (GMO) and legal issues impede its use. To the best of our knowledge, so far only two strains have been allowed for commercial implementation (Coulon et al., 2006; Husnik et al., 2006) although they are not extendedly used. In the European Union, Regulation (EC) 1829/2003 sets the legislation on genetically modified food and feed and postpone the use of GMO until better times.

4. Concluding Remarks

Wine making is characterized for being a microbiological driven process where the biological control is a requirement for safety, reproducibility, and consumer acceptance. Although the process is open to incorporate the new tools that have been developed in recent years, the winemaker and consumer reluctance to some of those novelties as well

as the reality of the cellars and the technological and expertise requirements of some of these methodologies makes their use in cellars still very limited. For instance, massive sequencing could be a very helpful methodology to monitor fermentations or post fermentative processes (aging, for instance) as well as safety control of the product. However, present development, costs, expertise, and timing makes their cellar application almost inviable. On the other hand, the use of CRISPR-Cas9 methodology that could generate mutant strains that incorporate wine making improvements (for instance reduction of urea and ethyl carbamate, increase of glycerol, reduced ethanol, improved aromatic expression) face the challenge of being considered GM and thus, the consumer rejection or the regulation limitation.

5. Acknowledgements

This manuscript has been prepared a within the framework of the SUMCULA and OENOBIO Erasmus + Programme Projects (2017-1-SE01-KA203-034570 and 2018-1-FR01-KA203-047839 respectively)

6. References

Alañón, M.E., Pérez-Coello, M.S., Marina, M.L., 2015. Wine science in the metabolomics era. TrAC Trends Anal. Chem. 74, 1–20.

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., Gírio, F., 2010. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. Appl. Microbiol. Biotechnol. 86, 965–972.

Alonso-del-Real, J., Pérez-Torrado, R., Querol, A., Barrio, E., 2019. Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization. Environ. Microbiol. 21, 1627–1644.

Alves, Z., Melo, A., Figueiredo, A.R., Coimbra, M.A., Gomes, A.C., Rocha, S.M., 2015. Exploring the *Saccharomyces cerevisiae* volatile metabolome: Indigenous versus commercial strains. PLoS One 10, e0143641.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J.M., Mas, A., 2010. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. Int. J. Food Microbiol. 144, 257-262.

Arapitsas, P., Guella, G., Mattivi, F., 2018. The impact of SO2 on wine flavanols and indoles in relation to wine style and age. Sci. Rep. 8, 858.

596 Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2012. The microbial ecology of wine
597 grape berries. Int. J. Food Microbiol. 153, 243-259
598 Barbosa, C., García-Martínez, J., Pérez-Ortín, J.E., Mendes-Ferreira, A., 2015.
599 Comparative transcriptomic analysis reveals similarities and dissimilarities in
600 *Saccharomyces cerevisiae* wine strains response to nitrogen availability. PLoS One
601 10, e0122709.
602 Bartowsky, E.J., 2009. Bacterial spoilage of wine and approaches to minimize it. Lett.
603 Appl. Microbiol. 48, 149–156.
604 Belda, I., Gobbi, A., Ruiz, J., de Celis, M., Ortiz-Álvarez, R., Acedo, A., Santos, A.,
605 2021. Microbiomics to Define Wine Terroir. In: Comprehensive Foodomics.
606 Cifuentes, A. (Ed.). Elsevier, 438-451. doi.org/10.1016/B978-0-08-100596-
607 5.22875-8
608 Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A.,
609 Moreno-Arribas, M., 2017. Microbial contribution to wine aroma and its intended
610 use for wine quality improvement. Molecules 22, 189.
611 Beltran, G., Torija, M.J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J.M., Rozès, N.,
612 Mas, A., 2002. Analysis of yeast populations during alcoholic fermentation: A six
613 year follow-up study. Syst. Appl. Microbiol. 25, 287–293.
614 Betlej, G., Bator, E., Oklejewicz, B., Potocki, L., Górka, A., Slowik-Borowiec, M.,
615 Czarny, W., Domka, W., Kwiatkowska, A., 2020. Long-term adaption to high
616 osmotic stress as a tool for improving enological characteristics in industrial wine
617 yeast. Genes (Basel). 11, 576.
618 Bisson, L.F., 2004. The Biotechnology of Wine Yeast. Food Biotechnol. 18, 63–96.
619 Bokulich, N.A., Collins, T.S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S.E.,
620 Millsa, D.A., 2016. Associations among wine grape microbiome, metabolome, and
621 fermentation behavior suggest microbial contribution to regional wine
622 characteristics. MBio. 7, e00631-16.
623 Bokulich, N.A., Ohta, M., Richardson, P.M., Mills, D.A., 2013. Monitoring Seasonal
624 Changes in Winery-Resident Microbiota. PLoS One. 8, e66437.
625 Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2014. Microbial
626 biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc.
627 Natl. Acad. Sci. 111, E139-E148.
628 Bordet, F., Joran, A., Klein, G., Roullier-Gall, C., Alexandre, H., 2020. Yeast-yeast
629 interactions: Mechanisms, methodologies and impact on composition.

630 Microorganisms 8, 600.
631 Branco, P., Viana, T., Albergaria, H., Arneborg, N., 2015. Antimicrobial peptides
632 (AMPs) produced by Saccharomyces cerevisiae induce alterations in the
633 intracellular pH, membrane permeability and culturability of Hanseniaspora
634 guilliermondii cells. Int. J. Food Microbiol. 205, 112–118.
635 Capozzi, V., Garofalo, C., Chiriatti, M.A., Grieco, F., Spano, G., 2015. Microbial terroir
636 and food innovation: The case of yeast biodiversity in wine. Microbiol. Res.
637 Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behaviour and metabolic
638 interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. 108,
639 239-245.
640 Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation.
641 Curr. Opin. Food Sci. 1, 1-6.
642 Coulon, J., Husnik, J.I., Inglis, D.L., van der Merwe, G.K., Lonvaud, A., Erasmus, D.J.,
643 van Vuuren, H.J.J., 2006. Metabolic Engineering of *Saccharomyces cerevisiae* to
644 Minimize the Production of Ethyl Carbamate in Wine. Am. J. Enol. Vitic. 57, 113
645 LP – 124.
646 Couto, J.A., Neves, F., Campos, F., Hogg, T., 2005. Thermal inactivation of the wine
647 spoilage yeasts *Dekkera/Brettanomyces*. Int. J. Food Microbiol. 104, 337–344.
648 Cozzolino, D., 2016. Metabolomics in Grape and Wine: Definition, Current Status and
649 Future Prospects. Food Anal. Methods 9, 2986–2997.
650 Curiel, J.A., Morales, P., Gonzalez, R., Tronchoni, J., 2017. Different non-
651 *Saccharomyces* yeast species stimulate nutrient consumption in *S. cerevisiae*
652 mixed cultures. Front. Microbiol. 8, 2121.
653 De Filippis, F., Parente, E., Ercolini, D., 2018. Recent Past, Present, and Future of the
654 Food Microbiome. Annu. Rev. Food Sci. Technol. 9, 589–608.
655 Dumont, M.G., Pommerenke, B., Casper, P., 2013. Using stable isotope probing to
656 obtain a targeted metatranscriptome of aerobic methanotrophs in lake sediment.
657 Environ. Microbiol. Rep. 5, 757–764.
658 Eldarov, M.A., Mardanov, A. V., 2020. Metabolic engineering of wine strains of
659 *Saccharomyces cerevisiae*. Genes (Basel). 11, 964.
660 Ferreira, D., Moreira, D., Costa, E., Silva, S., Pintado, M., Couto, J., 2013. The
661 Antimicrobial Action of Chitosan Against the Wine Spoilage Yeast
662 *Brettanomyces/Dekkera*. J. Chitin Chitosan Sci. 1, 240–245.
663 Fleet, G.H., 1993. Wine Microbiology and Biotechnology. Harwood Academic

664 Publishers, Chur, Switzerland.
665 Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11–
666 22.
667 Garde-Cerdán, T., López, R., Garijo, P., González-Arenzana, L., Gutiérrez, A.R.,
668 López-Alfaro, I., Santamaría, P., 2014. Application of colloidal silver versus sulfur
669 dioxide during vinification and storage of Tempranillo red wines. Aust. J. Grape
670 Wine Res. 20, 51–61.
671 Garofalo, C., El Khoury, M., Lucas, P., Bely, M., Russo, P., Spano, G., Capozzi, V.,
672 2015. Autochthonous starter cultures and indigenous grape variety for regional
673 wine production. J. Appl. Microbiol. 118, 1395-1408.
674 Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani,
675 M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in
676 simultaneous and sequential co-fermentation: A strategy to enhance acidity and
677 improve the overall quality of wine. Food Microbiol. 33, 271–281.
678 Godálová, Z., Kraková, L., Puškárová, A., Bučková, M., Kuchta, T., Piknová, Ľ.,
679 Pangallo, D., 2016. Bacterial consortia at different wine fermentation phases of
680 two typical Central European grape varieties: Blaufränkisch (Frankovka modrá)
681 and Grüner Veltliner (Veltlínske zelené). Int. J. Food Microbiol. 217, 110–116.
682 González-Jiménez, M. del C., Moreno-García, J., García-Martínez, T., Moreno, J.J.,
683 Puig-Pujol, A., Capdevilla, F., Mauricio, J.C., 2020. Differential analysis of
684 proteins involved in ester metabolism in two Saccharomyces cerevisiae strains
685 during the second fermentation in sparkling wine elaboration. Microorganisms 8,
686 403
687 Gonzalez, R., Quirós, M., Morales, P., 2013. Yeast respiration of sugars by non-
688 Saccharomyces yeast species: A promising and barely explored approach to
689 lowering alcohol content of wines. Trends Food Sci. Technol. 29, 55-61.
690 Goold, H.D., Kroukamp, H., Williams, T.C., Paulsen, I.T., Varela, C., Pretorius, I.S.,
691 2017. Yeast's balancing act between ethanol and glycerol production in low-
692 alcohol wines. Microb. Biotechnol. 10, 264–278.
693 Huang, L.H., Yuan, M.Q., Ao, X.J., Ren, A.Y., Zhang, H.B., Yang, M.Z., 2018.
694 Endophytic fungi specifically introduce novel metabolites into grape flesh cells in
695 vitro. PLoS One 13, e0196996
696 Husnik, J.I., Volschenk, H., Bauer, J., Colavizza, D., Luo, Z., van Vuuren, H.J.J., 2006.
697 Metabolic engineering of malolactic wine yeast. Metab. Eng. 8, 315–323.

698 Jakočiunas, T., Jensen, M.K., Keasling, J.D., 2016. CRISPR/Cas9 advances engineering
699 of microbial cell factories. Metab. Eng. 34, 44–59.
700 Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: Non-
701 *Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14, 215-
702 237.
703 Kioroglou, D., Lleixá, J., Mas, A., Portillo, M.C., 2018. Massive sequencing: A new
704 tool for the control of alcoholic fermentation in wine? Fermentation 4, 7.
705 Kioroglou, D., Mas, A., Portillo, M.C., 2020. High-Throughput Sequencing Approach
706 to Analyze the Effect of Aging Time and Barrel Usage on the Microbial
707 Community Composition of Red Wines. Front. Microbiol. 11, 2142.
708 Kutyna, D.R., Varela, C., Stanley, G.A., Borneman, A.R., Henschke, P.A., Chambers,
709 P.J., 2012. Adaptive evolution of *Saccharomyces cerevisiae* to generate strains
710 with enhanced glycerol production. Appl. Microbiol. Biotechnol. 93, 1175–1184.
711 Lisanti, M.T., Blaiotta, G., Nioi, C., Moio, L., 2019. Alternative Methods to SO_2 for
712 Microbiological Stabilization of Wine. Compr. Rev. Food Sci. Food Saf. 18, 455–
713 479.
714 Lleixà, J., Manzano, M., Mas, A., Portillo, M. del C., 2016a. *Saccharomyces* and non-
715 *Saccharomyces* competition during microvinification under different sugar and
716 nitrogen conditions. Front. Microbiol. 7, 1959.
717 https://doi.org/10.3389/fmicb.2016.01959
718 Lleixà, J., Martín, V., Portillo, M. del C., Carrau, F., Beltran, G., Mas, A., 2016b.
719 Comparison of fermentation and wines produced by inoculation of Hanseniaspora
720 vineae and Saccharomyces cerevisiae. Front. Microbiol. 7, 338.
721 López-Malo, M., Querol, A., Guillamon, J.M., 2013. Metabolomic Comparison of
722 Saccharomyces cerevisiae and the Cryotolerant Species S. bayanus var. uvarum
723 and S. kudriavzevii during Wine Fermentation at Low Temperature. PLoS One 8,
724 e60135.
725 Loureiro, V., Malfeito-Ferreira, M., 2003. Spoilage yeasts in the wine industry. Int. J.
726 Food Microbiol. 86, 23-50.
727 Mazzei, P., Spaccini, R., Francesca, N., Moschetti, G., Piccolo, A., 2013. Metabolomic
728 by 1H NMR spectroscopy differentiates “fiano di Avellino” white wines obtained
729 with different yeast strains. J. Agric. Food Chem. 61, 10816–10822.
730 McBryde, C., Gardner, J.M., de Barros Lopes, M., Jiranek, V., 2006. Generation of
731 Novel Wine Yeast Strains by Adaptive Evolution. Am. J. Enol. Vitic. 57, 423 LP –

430.

Medina, K., Boido, E., Dellacassa, E., Carrau, F., 2012. Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. Int. J. Food Microbiol. 157, 245-250.

Mencher, A., Morales, P., Valero, E., Tronchoni, J., Patil, K.R., Gonzalez, R., 2020. Proteomic characterization of extracellular vesicles produced by several wine yeast species. Microb. Biotechnol. 13, 1581–1596.

Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality and production. Food Res. Int. 43, 1844–1855.

Muysson, J., Miller, L., Allie, R., Inglis, D.L., 2019. The use of CRISPR-Cas9 genome editing to determine the importance of glycerol uptake in wine yeast during icewine fermentation. Fermentation 5, 93.

Navarro, Y., Torija, M.J., Mas, A., Beltran, G., 2020. Viability-PCR allows monitoring yeast population dynamics in mixed fermentations including viable but non-culturable yeasts. Foods 9, 1373.

Nissen, P., Arneborg, N., 2003. Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Arch. Microbiol. 180, 257–263.

Nookaew, I., Papini, M., Pornputtapong, N., Scalcinati, G., Fagerberg, L., Uhlén, M., Nielsen, J., 2012. A comprehensive comparison of RNA-Seq-based transcriptome analysis from reads to differential gene expression and cross-comparison with microarrays: A case study in *Saccharomyces cerevisiae*. Nucleic Acids Res. 40, 10084–10097.

Padilla, B., García-Fernández, D., González, B., Izidoro, I., Esteve-Zarzoso, B., Beltran, G., Mas, A., 2016. Yeast biodiversity from DOQ Priorat uninoculated fermentations. Front. Microbiol. 7, 930.

Padilla, B., Zulian, L., Ferreres, À., Pastor, R., Esteve-Zarzoso, B., Beltran, G., Mas, A., 2017. Sequential Inoculation of Native Non-*Saccharomyces* and *Saccharomyces cerevisiae* Strains for Wine Making. Front. Microbiol. 8, 1293.

Peng, C., Andersen, B., Arshid, S., Larsen, M.R., Albergaria, H., Lametsch, R., Arneborg, N., 2019. Proteomics insights into the responses of *Saccharomyces cerevisiae* during mixed-culture alcoholic fermentation with *Lachancea thermotolerans*. FEMS Microbiol. Ecol. 95, fiz126.

Peng, C., Viana, T., Petersen, M.A., Larsen, F.H., Arneborg, N., 2018. Metabolic

766 footprint analysis of metabolites that discriminate single and mixed yeast cultures
767 at two key time-points during mixed culture alcoholic fermentations.
768 Metabolomics 14, 93.
769 Petitgonnet, C., Klein, G.L., Roullier-Gall, C., Schmitt-Kopplin, P., Quintanilla-Casas,
770 B., Vichi, S., Julien-David, D., Alexandre, H., 2019. Influence of cell-cell contact
771 between *L. thermotolerans* and *S. cerevisiae* on yeast interactions and the exo-
772 metabolome. Food Microbiol. 83, 122–133.
773 Petruzzi, L., Capozzi, V., Berbegal, C., Corbo, M.R., Bevilacqua, A., Spano, G.,
774 Sinigaglia, M., 2017. Microbial Resources and Enological Significance:
775 Opportunities and Benefits. Front. Microbiol, 8, 995.
776 Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., Pinheiro, M.,
777 Egas, C., Gomes, A.C., 2015. Wine fermentation microbiome: A landscape from
778 different Portuguese wine appellations. Front. Microbiol. 6, 905.
779 Pretorius, I., 2016. Conducting Wine Symphonics with the Aid of Yeast Genomics.
780 Beverages 2, 36.
781 Pretorius, I.S., 2017. Synthetic genome engineering forging new frontiers for wine
782 yeast. Crit. Rev. Biotechnol. 37, 112–136.
783 Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-
784 *Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar
785 respiration. Int. J. Food Microbiol. 181, 85–91.
786 Raschmanová, H., Weninger, A., Glieder, A., Kovar, K., Vogl, T., 2018. Implementing
787 CRISPR-Cas technologies in conventional and non-conventional yeasts: Current
788 state and future prospects. Biotechnol. Adv. 36, 641–665.
789 Renault, P., Coulon, J., de Revel, G., Barbe, J.-C., Bely, M., 2015. Increase of fruity
790 aroma during mixed *T. delbrueckii*/*S. cerevisiae* wine fermentation is linked to
791 specific esters enhancement. Int. J. Food Microbiol. 207, 40–48.
792 Renouf, V., Claisse, O., Lonvaud-Funel, A., 2005. Understanding the microbial
793 ecosystem on the grape berry surface through numeration and identification of
794 yeast and bacteria. Aust. J. Grape Wine Res. 11, 316-327.
795 Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2006. Handbook of
796 Enology:The Microbiology of Wine and Vinifications, Handbook of Enology,
797 Volume 2. John Wiley & Sons.
798 Richter, C.L., Kennedy, A.D., Guo, L., Dokoozlian, N., 2015. Metabolomic
799 measurements at three time points of a chardonnay wine fermentation with

Saccharomyces cerevisiae. Am. J. Enol. Vitic. 68, 294-301.

Rizzotti, L., Levav, N., Fracchetti, F., Felis, G.E., Torriani, S., 2015. Effect of UV-C treatment on the microbial population of white and red wines, as revealed by conventional plating and PMA-qPCR methods. Food Control 47, 407–412.

Roca-Mesa, H., Sendra, S., Mas, A., Beltran, G., Torija, M.J., 2020. Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. Microorganisms 8, 157.

Rojo-Bezares, B., Sáenz, Y., Zarazaga, M., Torres, C., Ruiz-Larrea, F., 2007. Antimicrobial activity of nisin against *Oenococcus oeni* and other wine bacteria. Int. J. Food Microbiol. 116, 32–36.

Rossouw, D., Bagheri, B., Setati, M.E., Bauer, F.F., 2015. Co-flocculation of yeast species, a new mechanism to govern population dynamics in microbial ecosystems. PLoS One 10, e0136249.

Roullier-Gall, C., David, V., Hemmler, D., Schmitt-Kopplin, P., Alexandre, H., 2020. Exploring yeast interactions through metabolic profiling. Sci. Rep. 10, 6073.

Ruiz-Moreno, M.J., Raposo, R., Moreno-Rojas, J.M., Zafrilla, P., Cayuela, J.M., Mulero, J., Puertas, B., Guerrero, R.F., Piñeiro, Z., Giron, F., Cantos-Villar, E., 2015. Efficacy of olive oil mill extract in replacing sulfur dioxide in wine model. LWT - Food Sci. Technol. 61, 117–123.

Sadoudi, M., Rousseaux, S., David, V., Alexandre, H., Tourdot-Maréchal, R., 2017. Metschnikowia pulcherrima influences the expression of genes involved in PDH bypass and glyceropyruvic fermentation in *Saccharomyces cerevisiae*. Front. Microbiol. 8, 1137.

Sandberg, T.E., Salazar, M.J., Weng, L.L., Palsson, B.O., Feist, A.M., 2019. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. Metab. Eng. 56, 1–16.

Santos, M.C., Nunes, C., Saraiva, J.A., Coimbra, M.A., 2012. Chemical and physical methodologies for the replacement/reduction of sulfur dioxide use during winemaking: Review of their potentialities and limitations. Eur. Food Res. Technol. 234, 1-12.

Shekhawat, K., Patterton, H., Bauer, F.F., Setati, M.E., 2019. RNA-seq based transcriptional analysis of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed-culture fermentations under anaerobic conditions. BMC Genomics 20, 145.

834 Silva, V., Igrejas, G., Falco, V., Santos, T.P., Torres, C., Oliveira, A.M.P., Pereira, J.E.,
835 Amaral, J.S., Poeta, P., 2018. Chemical composition, antioxidant and antimicrobial
836 activity of phenolic compounds extracted from wine industry by-products. Food
837 Control 92, 516–522.
838 Sirén, K., Mak, S.S.T., Fischer, U., Hansen, L.H., Gilbert, M.T.P., 2019a. Multi-omics
839 and potential applications in wine production. Curr. Opin. Biotechnol. 56, 172–
840 178.
841 Sirén, K., Mak, S.S.T., Melkonian, C., Carøe, C., Swiegers, J.H., Molenaar, D., Fischer,
842 U., Thomas P Gilbert, M., 2019b. Taxonomic and functional characterization of
843 the microbial community during spontaneous in vitro fermentation of riesling
844 must. Front. Microbiol. 10, 697.
845 Steensels, J., Snoek, T., Meersman, E., Nicolino, M.P., Voordeckers, K., Verstrepen,
846 K.J., 2014. Improving industrial yeast strains: exploiting natural and artificial
847 diversity. FEMS Microbiol. Rev. 38, 947–995.
848 Stefanini, I., Cavalieri, D., 2018. Metagenomic approaches to investigate the
849 contribution of the vineyard environment to the quality of wine fermentation:
850 Potentials and difficulties. Front. Microbiol. 9, 991.
851 Sternes, P.R., Lee, D., Kutyna, D.R., Borneman, A.R., 2017. A combined meta-
852 barcoding and shotgun metagenomic analysis of spontaneous wine fermentation.
853 Gigascience 6, gix040.
854 Suárez, R., Suárez-Lepe, J.A., Morata, A., Calderón, F., 2007. The production of
855 ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A
856 review. Food Chem. 102, 10–21.
857 Sunyer-Figueres, M., Vázquez, J., Mas, A., Torija, M.J., Beltran, G., 2020.
858 Transcriptomic insights into the effect of melatonin in *Saccharomyces cerevisiae*
859 in the presence and absence of oxidative stress. Antioxidants 9, 947.
860 Torija, M.J., Rozès, N., Poblet, M., Guillamón, J.M., Mas, A., 2001. Yeast population
861 dynamics in spontaneous fermentations: Comparison between two different wine-
862 producing areas over a period of three years. Antonie Van Leeuwenhoek 79, 345–
863 352. h
864 Tronchoni, J., Curiel, J.A., Morales, P., Torres-Pérez, R., Gonzalez, R., 2017. Early
865 transcriptional response to biotic stress in mixed starter fermentations involving
866 *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. Int. J. Food Microbiol.
867 241, 60–68.

868 Valera, M.J., Sainz, F., Mas, A., Torija, M.J., 2017. Effect of chitosan and SO2 on
869 viability of *Acetobacter* strains in wine. Int. J. Food Microbiol. 246, 1–4. h
870 Van Wyk, N., Grossmann, M., Wendland, J., Von Wallbrunn, C., Pretorius, I.S., 2019.
871 The Whiff of Wine Yeast Innovation: Strategies for Enhancing Aroma Production
872 by Yeast during Wine Fermentation. J. Agric. Food Chem. 67, 13496–13505.
873 van Wyk, N., Kroukamp, H., Espinosa, M.I., von Wallbrunn, C., Wendland, J.,
874 Pretorius, I.S., 2020. Blending wine yeast phenotypes with the aid of CRISPR
875 DNA editing technologies. Int. J. Food Microbiol. 324, 108615.
876 Varela, C., Bartel, C., Onetto, C., Borneman, A., 2020. Targeted gene deletion in
877 *Brettanomyces bruxellensis* with an expression-free CRISPR-Cas9 system. Appl.
878 Microbiol. Biotechnol. 104, 7105–7115.
879 Vigentini, I., Gebbia, M., Belotti, A., Foschino, R., Roth, F.P., 2017. CRISPR/Cas9
880 system as a valuable genome editing tool for wine yeasts with application to
881 decrease urea production. Front. Microbiol. 8, 2194.
882 Wang, C., Mas, A., Esteve-Zarzoso, B., 2016. The Interaction between *Saccharomyces*
883 *cerevisiae* and Non-*Saccharomyces* Yeast during Alcoholic Fermentation Is
884 Species and Strain Specific. Front. Microbiol. 7, 502.
885 Weninger, A., Fischer, J.E., Raschmanová, H., Kniely, C., Vogl, T., Glieder, A., 2018.
886 Expanding the CRISPR/Cas9 toolkit for *Pichia pastoris* with efficient donor
887 integration and alternative resistance markers. J. Cell. Biochem. 119, 3183–3198.
888 Wu, D., Xie, W., Li, X., Cai, G., Lu, J., Xie, G., 2020. Metabolic engineering of
889 *Saccharomyces cerevisiae* using the CRISPR/Cas9 system to minimize ethyl
890 carbamate accumulation during Chinese rice wine fermentation. Appl. Microbiol.
891 Biotechnol. 104, 4435–4444.
892 Yang, M.Z., Ma, M. Di, Yuan, M.Q., Huang, Z.Y., Yang, W.X., Zhang, H.B., Huang,
893 L.H., Ren, A.Y., Shan, H., 2016. Fungal endophytes as a metabolic fine-tuning
894 regulator for wine grape. PLoS One 11, e0163186.
895 Zarraonaindia, I., Owens, S.M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax,
896 S., Bokulich, N.A., Mills, D.A., Martin, G., Taghavi, S., van der Lelie, D., Gilbert,
897 J.A., 2015. The soil microbiome influences grapevine-associated microbiota. MBio
898 6, e02527-14.
899 Zepeda-Mendoza, M.L., Edwards, N.K., Madsen, M.G., Abel-Kistrup, M., Puetz, L.,
900 Sicheritz-Ponten, T., Swiegers, J.H., 2018. Influence of *Oenococcus oeni* and
901 *Brettanomyces bruxellensis* on wine microbial taxonomic and functional potential

902 profiles. Am. J. Enol. Vitic. 69, 321–333.
903 Zhang, Yueping, Wang, J., Wang, Z., Zhang, Yiming, Shi, S., Nielsen, J., Liu, Z., 2019.
904 A gRNA-tRNA array for CRISPR-Cas9 based rapid multiplexed genome editing in
905 *Saccharomyces cerevisiae*. Nat. Commun. 10, 1053.
906 Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., Nury, F.S., 1995. Sorbic Acid, Benzoic
907 Acid, and Dimethyldicarbonate BT - Wine Analysis and Production, in:
908 Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., Nury, F.S. (Eds.). Springer US,
909 Boston, MA, pp. 209–215.
910

Legends to Figures:

Figure 1: Schematic representation of the workflow followed during metagenomic and metatrascriptomic analysis from different wine-related samples. Within metabolomics, the comparison between metabarcoding and shotgun sequencing is presented, whereas the metatranscriptomics indicates the main differences between microarrays and RNAseq outputs.

Figure 2: Inoculated and spontaneous alcoholic fermentations.